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## ELECTROPHORESIS OF TOBACCO MOSAIC VIRUS[1]


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### INTRODUCTION

Iwanowski's[34] demonstration in 1892 that the mosaic disease of tobacco is caused by a filter-passing agent opened a new and extensive field of research in the pathology of both plants and animals. Since then a number of different virus[4] diseases have been found affecting a variety of plants and animals. The bacteriophage has also been considered a virus by many workers.

Much literature dealing with various phases of virus diseases has been published. In the following review we have attempted to abstract only the work giving evidence as to the nature of the viruses.

### REVIEW OF LITERATURE

*Cytological Studies.*—For some time pathologists resorted to cytological methods in their search for a visible parasite causing the virus diseases. A great deal of careful work has been done in this field. Iwanowski,[35] Dickson,[21] McKinney, Eckerson and Webb,[45] Goldstein,[30] Palm,[54] Rawlins and Johnson,[58] Hoggan,[32] Kunkel,[38] Holmes,[33] Smith,[61] and various students of animal pathology have described abnormal intracellular bodies in virus-infected host tissues. The structure of these bodies is not sufficiently distinct to warrant the conclusion that they are a stage of a causal organism. In fact, many of the workers are inclined to the view that they are products of the causal agent or of the diseased host cell.


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[4] In this paper a virus is considered to be a filter-passing agent capable of causing an infectious disease.

*Size of Virus Particles.*—Iwanowski[34] and Beijerinck[8] discovered independently that the infective agent of tobacco mosaic passed through filters which intercepted ordinary bacteria. Beijerinck assumed that the virus was in the form of a *"contagium vivum fluidum."* Since then the size limits of tobacco mosaic virus particles have been studied by Duggar and Karrer.[23] Using a series of ultrafilters graded by means of organic particles of known dimension the size of the virus particles was found to be comparable to that of haemoglobin, having a diameter of approximately 30$\mu\mu$.

Olitsky, Traum and Schoening[52] employing similar methods estimated the size of the virus particles of foot and mouth disease to be somewhere between 20 and 100$\mu\mu$.

Prausnitz,[55] using similar technique estimated the size of Flexner bacteriophage as approximately 20$\mu\mu$.

From the above results it appears that we cannot hope to observe the virus particles by ordinary microscopic methods. The presence of colloidal particles of approximately this size has been detected by use of the ultramicroscope in the hands of students of colloidal chemistry. However, until we are able to separate a virus from the colloids found in plant juices or animal fluids we cannot hope to detect or distinguish the virus particles by this method. Numerous attempts have been made to detect motile organisms in virus-infected fluids by means of the dark-field microscope but in general such attempts have been unsuccessful.

*Dilution.*—It is known that the virus of tobacco mosaic withstands great dilution without losing its infective properties. Allard[2] showed that infective tobacco juice diluted one part to a thousand is quite as effective in producing the disease as the undiluted juice, but that when diluted one part to ten thousand the percentage of infection is reduced. In some cases positive results were found even in dilutions as high as one part to one million. These results indicate that the concentration of virus in plant juice is very high.

*Longevity.*—Tobacco mosaic virus can be dried for a relatively long period without losing its disease-inciting properties. Chapman[16] finds 3 years, Beijerinck[8] and Walker[67] 2 years, and Allard[1] 18 months as its period of longevity.

There is considerable difference in the longevity of other viruses causing plant diseases. Walker[67] reports that tomato mosaic virus when dried at air temperature remained active 30 days, ground cherry virus 23 days, and cucumber mosaic virus less than 24 hours. However, when these viruses were kept in their plant extracts some of them remained infectious for a much longer period of time, tomato mosaic

virus remaining infectious 138 days, ground cherry virus 69 days, and cucumber mosaic virus from 24 to 48 hours.

Hastings[31] reports that spores of anthrax bacillus remained viable for 18.5 years in a naturally infected sample of pond water. This period is about forty-two times as great as that noted by previous observers working with spores in artificial culture. "A member of the colon aerogenes group" isolated from a corn plant and then placed on filter paper kept at 37° C remained viable 31 days. The same organism when left on corn stover withstood desiccation for a period of 7 years. Thus it is evident that certain bacteria are even more resistant to drying than are the viruses.

*Cultivation of Virus.*—Although the viruses causing mosaic diseases multiply very rapidly in living plant tissues they have never been satisfactorily cultivated in artificial media.

Olitsky[50] used fresh juice from healthy tomato plants as a medium and produced infection up to the twelfth subplant. "By the fourth subplant the original subplant was present in an estimated dilution of one to ten million." This exceeds the dilution limit of all known viruses. From these results he concluded that he had cultivated the virus in an artificial medium.

Following the publication of Olitsky's findings four other investigators, Mulvania,[46] Goldsworthy,[29] Purdy,[56] and Smith[62] repeated his experiments in an attempt to confirm his results. All their results were negative, however.

Purdy[57] demonstrated that tobacco mosaic virus multiplies in freshly detached tobacco leaves placed petiole downward in moist sand. Under such conditions symptoms do not appear.

*Thermal Inactivation.*—As compared with the vegetative stage of most microorganisms certain viruses have a relatively high thermal inactivation point. In this property these viruses are similar to enzymes. Bertrand and Thomas[10] state that, "'Nearly all enzymes are completely inactivated by short exposure to a temperature of 100° C.'" The inactivation of saccharase and blood catalase has been found by Willstatter, Graser and Kuhn[68] to be in a great measure dependent on the degree of purity of the given enzyme.

Mulvania[47] reports that a temperature of 80° C for three days reduced the activity of tobacco mosaic virus 40 per cent.

Allard's work[3] indicates that a temperature of 90° C or above for five minutes inactivated the virus.

The thermal death-point of the vegetative stage of most microorganisms lies between 37° and 55° C. Some of the viruses also have thermal inactivation points in this range. Esty[26] found that resistant

spores of *Bacillus botulinus* under optimum conditions of growth withstood a temperature of 100° C for 330 minutes, 110° C for 33 minutes and 120° C for 4 minutes.

It was found by McKinney[44] that the thermal inactivation point was depressed approximately 6° C when infective juice of mosaic tobacco plants was diluted one part to one hundred.

Air-dried mosaic tobacco leaves were found by Allard[3] to retain their infectivity after being heated at 100° C for one-half hour, while infective extract which had been evaporated to dryness was easily inactivated by this treatment. These results indicate that the system in which the virus is contained has a distinct influence upon its thermal inactivation point.

Allard[3] has demonstrated that low temperatures do not inactivate the virus of tobacco mosaic, —12° C for one to four hours or repeated freezing and thawing failing to inactivate it. Even the temperature of liquid air, —180° C, did not inactivate it.

Johnson's work[36] indicated that optimum temperature for the development of mosaic symptoms in tobacco is similar to that which is optimum for bacterial growth, namely around 30° C. This temperature is lower than that most favorable for the activity of most enzymes, the optimum temperature for enzymes, according to Waksman and Davison,[66] usually falling between 40° and 50° C. At 35° C symptoms of tobacco mosaic failed to develop.

Johnson's results are very interesting but unfortunately are open to several interpretations. It is conceivable that the metabolism of the host plant may have been altered sufficiently by the high temperature to produce conditions in the host cells which are unfavorable for the rapid multiplication of the virus and that inhibition of symptoms may not have been due to the direct influence of high temperature on the virus.

In general, it appears that temperature studies have yielded little evidence as to the nature of the viruses.

*Action of Chemicals.*—Viruses in general are relatively resistant to chemicals which are toxic to most organisms. Allard[1, 3] found that common disinfectants such as phenol, cresol, thymol, camphor and naphthalene have little effect on tobacco mosaic virus. Even formaldehyde below concentrations of 4 per cent did not readily inactivate the virus. Salts of heavy metals such as lead nitrate and mercuric chloride had little effect on certain viruses, a 1 per cent solution of mercuric chloride failing to inactivate tobacco mosaic virus in 33 days. Strong acids such as nitric and hydrochloric acids reduced infectivity only in concentration approaching 1 gram in 50 to 100 cc

of infective juice. Phosphoric, citric and acetic acids were without effect except in concentrations of 1 gram in 20 to 50 cc of infective juice. Hydroxides of aluminum and nickel tended to form precipitates and leave the supernatent liquid without infectious properties. In the case of aluminum hydroxide the virus was found to be in the precipitate and was recovered from it. It is interesting to note here that aluminum hydroxide is a positive colloid and would be expected to precipitate negative colloids. Nickel ions seemed to have a definite toxic effect upon the infective principle, destroying it entirely. In this solution nickel hydroxide was present in a concentration of 2 grams in 1000 cc of infective juice. When juice from mosaic tobacco plants was passed through talc or kaolin the filtrate was found to be free of virus.

The American Commission for the Investigation of the Foot and Mouth Disease,[52] working with the virus of the foot-and-mouth disease of animals, found that sodium hydroxide had no coagulating action on the lymph containing the virus and reported it as being virucidal in concentrations of 1 to 2 per cent acting for 1 minute.

Allard[3] found that 1 gram of sodium hydroxide in 1000 cc of virus solution inactivated tobacco mosaic virus after 2 days.

He further found that green mosaic tobacco leaves are quickly killed by ether and chloroform vapor but the infective principle contained in them is uninjured. Toluol and carbon tetrachloride are also without effect on the virus.

Allard showed that ethyl alcohol added to filtered plant extract containing tobacco mosaic virus throws down a precipitate carrying the virus with it. When alcohol of 45 to 50 per cent strength was used the supernatant liquid was free of virus. However, higher concentrations, 75 to 80 per cent, inactivated the virus in the precipitate.

The virucidal action of alcohol on the same virus taken from mosaic tomato was investigated by Smith.[62] The virus was not inactivated by alcohol at as high a concentration as 90 per cent. The supernatant liquid remained active after treatment with 70 per cent alcohol and the residue still retained its full activity after subjection to 90 per cent alcohol. Even when precipitation was prevented by the addition of sodium hydroxide, 90 per cent alcohol failed to inactivate the virus.

Vinson and Petre[65] find that precipitation of the virus from the juice of mosaic tobacco plants is fairly complete when two volumes of acetone or alcohol are added to one volume of infective extract. Such a precipitate was found to be highly infective when suspended in water.

Olitsky, Traum and Schoening[52] indicated that the action of alcohol on the foot-and-mouth virus is largely prevented by the coagulum formed around the virus particles. They showed that filtered lymph containing active virus is less resistant to alcohol than the unfiltered lymph and regard this difference as due to the smaller amount of protein present in the former. When coagulation by alcohol was prevented by the addition of a small amount of sodium hydroxide the alcohol was found to have a greater virucidal action.

On the other hand, Bronfenbrenner[13] working with bacteriophage gave evidence that the apparent inactivation of a bacteriophage by alcohol is a surface phenomenon rather than that of disinfection by the penetration of the alcohol into an organism. He has demonstrated that in the case of an unpurified bacteriophage the major portion of the lytic principle is found in the precipitate formed on the addition of alcohol. Bronfenbrenner then purified the bacteriophage as far as possible by growing the bacteria on a synthetic medium with ammonium salts as the only source of nitrogen. A lytic filtrate of very high potency was obtained. This filtrate was then fractionated and freed of all dialyzable material by means of electro ultra-filtration. After hydrolysis a residue was obtained which appeared to be free from ammonia when tested with Nessler's reagent. This indicates the absence of proteins in the bacteriophage. When the purified bacteriophage was treated with 10 volumes of alcohol precipitation did not occur and no inactivation of the lytic principle took place after remaining in this solution at 22° to 25° C for eight days. Thus it appears that in this case the alcohol has little direct influence on the virus.

Duggar and Armstrong[24] found that expressed juice from healthy pokeweed plants is capable of inactivating the virus of tobacco mosaic. They suggest that the action is probably due to the adsorption of the virus by the colloids of the foreign plant juice.

Elmer[25] found that extracted juice from healthy bean, cucumber, and tobacco plants reduced the infective power of tomato mosaic. This effect, however, was only temporary, full infectivity being regained in 21 days.

The results of the above workers show that viruses may resist certain powerful disinfectants but are inactivated by numerous relatively inert colloids. Such results indicate that colloids in the medium may be of great importance in inactivating the viruses or in their influence on inactivation by disinfectants. It is apparent, therefore, that a virus must be purified before its reaction to various chemicals can be determined with certainty.

The relatively great resistance of certain viruses to toxic chemicals is apparently evidence favoring their non-living nature.

*Ultra-Violet Radiation.*—Bovie[11] showed that ultra-violet radiation of wave length shorter than 292.5$\mu\mu$ killed bacteria and spores of fungi in 10 minutes.

It was found by Smith[61] that although *Bacillus prodigiosus* suspended in distilled water is inactivated by ultra-violet light in 30 seconds, tobacco mosaic virus in filtered plant extract is permanently inactivated only after continuous exposure for 30 minutes to such light. As shown by the following workers the differential absorption of the effective rays by the two media, distilled water and plant extract, may have been responsible for at least part of the difference observed by Smith.

Arthur and Newell[5] used samples of purified virus prepared by the acetone precipitation method outlined by Vinson and Petre.[65] Drops of the purified tobacco mosaic virus were distributed on a glass plate and covered with a quartz plate about ⅛ inch thick. The irradiation took place through the quartz plate at a distance of 15 inches. Such a preparation of tobacco mosaic virus was found to be completely inactivated by 15 seconds exposure to the open arc. Virus applied to surface of tobacco leaves could be inactivated by ultra-violet radiation but after the virus had penetrated the plant, inactivation could not be made to take place.

McKinley, Fisher and Holden[43] studied the influence of ultra-violet light on bacteria, a bacteriophage, and viruses. They state that under the conditions of their experiments, "a lytic principle active for *Bacterium coli* 'D' is acted upon by ultra-violet light in much the same way as are two strains of known filterable viruses, i.e., herpes and Levaditi's so-called encephalitis virus. Exposure to ultra-violet light at a distance of one foot for forty minutes is sufficient to attenuate or destroy both the bacteriophage and the two filterable viruses employed in these experiments." *B. coli*, however, was apparently unaffected under the same conditions. In a substrate of normal rabbit serum the bacteriophage and the two filterable viruses are protected from the action of ultra-violet light.

Baker and Nanavutty[6] considered the opacity of beef broth to ultra-violet rays in studying the effect of such rays upon bacteriophage. They found that beef broth in the proportion of 1:1000 in normal saline produced inappreciable opacity to the bactericidal light rays when the depth of the exposed solution was less than 1 cm. Because a quantitative comparison of the results obtained by various workers is difficult due to differences in methods and lack of a unit

measurement of the bactericidal output of a mercury vapor lamp these investigators took the value 1 as time required to destroy *B. coli* and made relative measurements of the inactivating effect of ultra-violet rays upon several bacteriophages and enzymes. Their results are as follows: *B. coli* 1, Shiga bacteriophage about 2, staphylo bacteriophage 1 to 2, trypsin and complement 20 to 30, haemolytic amboceptor 50, diastase and lysozyme 120. From this they conclude that the susceptibility of bacteriophage to ultra-violet rays is similar to that of bacteria.

Because of the different results obtained by the above workers no general conclusions can be drawn as to the relative resistance of organisms, viruses, and enzymes to ultra-violet light.

*The Influence of X-Rays on Viruses.*—Mulvania[47] found that X-rays do not inactivate tobacco mosaic virus but that attenuation results when the exposure is lengthened.

*Attenuation.*—It has been observed by many virus investigators that attenuation of viruses may take place under certan conditions. Mayer[42] found that continued heating at 60° C did not change perceptibly the infective power of tobacco mosaic virus but subjection to a temperature of 65° to 75° C caused attenuation.

Other studies on the attenuation of tobacco mosaic virus were made by Johnson.[37] Virus-inoculated tobacco plants were placed in a constant temperature chamber at a temperature between 35° and 37° C for ten or more days. At this temperature mosaic symptoms are wholly or partially masked. Virus from these plants produced a mild form of the disease in inoculated plants. Repeated serial transfers from such plants to healthy tobacco plants did not perceptibly alter the attenuated condition of the virus.

Carsner,[15] working with the virus causing curly top of sugar beet, reports that the passage of this virus through *Chenopodium murale, Rumex crispus* and *Suaeda moquini* causes the virus to become attenuated so that a mild form of curly top develops when the virus is transferred to healthy beets.

Organisms are known to be attenuated by certain unfavorable conditions. Consequently this behavior may be regarded as evidence of the living nature of a virus.

*Respiration.*—Carbon dioxide is produced during respiration in most organisms. Bronfenbrenner[13] by the aid of a sensitive micro-respirometer attempted to detect the generation of $CO_2$ by a bacteriophage and by the herpes and rabies viruses. After being confined for 48 hours in the apparatus under both anaerobic and aerobic conditions neither bacteriophage nor viruses gave a positive test for the presence

of carbon dioxide. From these results it appears that the virus either has a very unusual type of metabolism in which carbon dioxide is not given off, as is reported by Palladin[53] for acetic acid and glycerine bacteria and for sorbose bacteria by Bertrand,[9] that respiration is so slight that the carbon dioxide is not detectable; or a third alternative, that the virus is non-living and therefore does not respire.

*Influence of Oxygen.*—Johnson[36] finds that the longevity of tobacco mosaic virus is shorter in well-aerated sand and sandy soils than in heavier clay and organic soils. When exposed in a moist condition to an excess of oxygen the virus is sensitive to its inactivating influence. Cleveland[18] has shown similar behavior in protozoa. On the other hand, Bayliss[7] states that the enzyme rennet can be inactivated by passing air through the solution.

*Purification of Virus.*—It seems clear that in some cases the true properties of the viruses have not been determined, the results having been greatly influenced by the constituents of the medium. A knowledge of this relation of the virus properties to environment makes apparent the importance of purifying the viruses as much as possible before attempting to determine their properties.

Arnold and Weiss[4] separated a bacteriophage, lytic to *Bacillus typhosus*, from antigenic bacterial proteins derived from dissolved bacteria. A precipitation of antigenic bacterial proteins was effected by adding a 14 per cent solution of $Na_2SO_4$. Digestion with trypsin was also found to give satisfactory results. A 1 per cent trypsinized bacteriophage solution was incubated for 48 hours, filtered through paper and then passed through a Berkefeld candle. The absence of antigenic bacterial proteins was indicated by the failure of the supernatant liquid and filtrate to produce agglutinins against the corresponding bacteria.

Bronfenbrenner[13] and De Groat[20] also purified bacteriophages until no test for proteins was obtained.

Among investigators of viruses causing plant diseases Vinson and Petre[65] have attempted to purify tobacco mosaic virus by using a number of different methods.

They succeeded in removing some of the phosphates, sulfates, and most of the proteins and pigments from the extract by using low concentrations of lead acetate and barium acetate. Safranin was found to be very effective in precipitating the virus, 100 cc of a 1 per cent safranin solution added to 50 cc of infective juice causing a precipitate to form and leaving a supernatant liquid almost free of virus. The virus was then released from the safranin by adding amyl alcohol. Attempts were also made to salt out the virus by means of ammonium

sulfate. The precipitate, however, contained salts, proteins, and pigments as well as the virus.

Heat fractionation was tried. Juice from mosaic Turkish tobacco was separated into two heat-precipitable fractions, one around 85° C and the other above 90° C. The first fraction contained an appreciable amount of nitrogen and was still infective after the heating, although the virus concentration was greatly reduced. Boiling the juice rendered it non-infectious.

Brewer, Kraybill, and Gardner[12] attempted to purify tomato mosaic virus by centrifuging and clearing with aluminum gel. They succeeded in obtaining a very clear and active preparation of the virus.

Sherman, Caldwell, and Adams[59] purified pancreatic amylase until it was very active in dilutions as great as 1:100,000,000. They consider that even the most delicate protein test cannot detect proteins in concentrations less than 1:10,000 and that the failure of certain workers to obtain a protein test from their purified enzyme preparations may be due to the very low concentration of enzyme in the solutions tested. It is possible that the above criticism may be applicable to work on the viruses. Certainly failure to detect proteins in a purified virus or bacteriophage should not be considered as proof of the absence of proteins unless a very concentrated preparation of virus or bacteriophage has been used.

*Electrophoresis.*—In their experiments on electrophoresis, Olitsky, Traum, and Schoening[52] found an isoelectric range of the foot-and-mouth virus around pH 8; below pH 8 the virus carried a positive charge and above this pH a negative charge. Thus the virus appeared to behave like a protein having an isoelectric point around pH 8.

Todd[64] demonstrated that a bacteriophage lytic to *Bacillus dysenteria* Shiga isolated from chicken droppings migrated to the anode between pH 3.6 and 7.6. He failed to find an isoelectric point as the phage showed signs of inactivation at lower pH values.

Douglas and Smith[22] found that vaccinia virus migrates to the anode in the range pH 5.5 to 8.4. They further found that the isoelectric point of the tissue protein was pH 6.8. Therefore, on the alkaline side of its isoelectric point the protein migrated with the virus to the anode but on its acid side the protein migrated to the cathode.

By electrophoresis Olitsky and Long[51] obtained active virus at the anode from the serum of vaccinia-recovered rabbits. This serum had given no infection when used as an inoculum before electrophoresis. They believe that electrophoresis concentrated the virus at the anode.

Vinson and Petre[65] reported that the virus of tomato mosaic migrated to the negative electrode at pH 4.76.

Until the electrophoresis of more viruses is studied we cannot draw conclusions as to the similarity of their electrophoretic behavior or as to the significance of such behavior.

The migration of bacteria in an electric field has been studied by many investigators. Winslow, Falk, and Caulfield[69] found *Bacillus cereus* to be isoelectric at pH 3, positively charged between pH 3 and 1, and isoelectric at pH 1.

Winslow and Shaughnessy[70] found one isoelectric point of *Bacillus cereus* and *Bacterium coli* near pH 3.0 and another around pH 13.5.

*Pneumococcus* (variant type 1) was shown by Falk and Jacobson[27] to be charged negatively above pH 3.3 and positively between pH 2.7 and pH 0. At pH 0 another isopotential point was approached.

Falk, Sharp, and Link[28] found that bacteria from the smooth colonies of *Bacterium phaseoli sojense* were positively charged between pH 1.4 and pH 2.8 and negatively charged between pH 3.1 and 10. Bacteria from rough colonies showed a positive charge between pH 1.2 and 2.6 and a negative charge above pH 2.8.

Northrup and de Kruif[48] found that the bacillus of rabbit septicemia (type D) had a negative charge above pH 3, was isoelectric at pH3, and had a positive charge at pH 2.2. Another isoelectric point was observed around pH 1.1. Another strain (type G) was isoelectric around pH 4.2 and was positively charged at pH 2, which was the lowest pH value tested. *Bacillus typhosus* was found to be isoelectric around pH 3, positively charged from pH 3 to around pH 1, and isoelectric below pH 1.

Addition of egg albumin or globin to the bacillus of rabbit septicema was found by Northrup and de Kruif[49] to change the isoelectric point of this organism to approximately that of the added protein.

In general it appears that bacteria usually have a negative charge at pH values between 3 and such high values as 13. An isoelectric point is commonly found around pH 3 and the organisms usually have a positive charge in the region between pH 3 and 1. Around pH 1 another isoelectric point has usually been found.

When having a positive charge the organisms usually migrate toward the cathode at a comparatively slow rate, indicating a low potential at the surface of the organisms. The most rapid movement toward the anode is usually at higher pH values. Rapid migration toward this pole is usually observed between pH 6 and 9.

All of the studies on electrophoresis of viruses have been made at relatively high pH values. Further studies should be made at low pH values in order to compare the electrophoretic behavior of viruses with bacteria at pH values where the bacteria show characteristic changes.

Since Northrup and de Kruif[49] have shown that the addition of sufficient protein confers the electrophoretic behavior of the protein on bacteria, and Loeb[40] has shown a similar action of proteins on negative colloids such as collodion particles, one may well suspect that a virus which shows electrophoretic behavior similar to a protein is exhibiting the properties of adsorbed protein rather than those of the virus.

*Summary of Available Evidence on the Nature of the Viruses.*— Most of the studies made on virus properties have not yielded strong evidence as to whether the viruses studied were living or inanimate. It is of course possible that all of the viruses are not similar in nature. In fact the so-called "incubation periods" observed in the transmitting insect and host plant by Smith and Boncquet[63] and Severin[60] in the case of curly top of sugar beet, and by Kunkel[39] in the insect transmitting aster yellows suggests that these viruses may be heteroxenous organisms and may be quite different from most of the mosaic viruses.

Perhaps the strongest evidence favoring the theory that the mosaic viruses are organisms is furnished by their susceptibility to attenuation, while the strongest evidence favoring their inanimate nature is apparently their great resistance to certain chemicals. The apparent absence of protein in the bacteriophage may also be considered as evidence against the phage being a living organism.

The most promising type of work for gaining evidence on the nature of the viruses would appear to be an attempt at purification of the viruses and a study of the properties of the purified viruses. Bronfenbrenner,[14] Vinson and Petre,[65] and Brewer, Kraybill, and Gardner[12] have already begun this type of work and have made considerable progress.

Electrophoresis should be very helpful in purifying a virus and in giving evidence as to its nature.

If electrophoretic behavior of a virus at different pH values were known it should be possible to separate the virus from much of the colloidal material in the plant juice by causing the migration of the virus to one pole while much of the other colloidal material fails to move or migrates to the opposite pole. For example the bacteriophage

studied by Todd which had a negative charge at pH 3.6 would migrate to the anode at this pH while most, if not all, of the proteins in solution would migrate to the cathode, since most proteins have an isoelectric point above this pH.

With the exception of the single observation made by Vinson and Petre,[65] no studies on the electrophoresis of viruses causing plant diseases have been reported. It appeared to us that a study of the electrophoretic behavior of plant viruses might yield evidence as to the nature of such viruses, and as to their similarity to bacteria and to the viruses causing animal diseases. Furthermore, such a study of the electrophoretic behavior of a virus at various pH values is necessary if one is to proceed intelligently in an attempt to use electrophoresis or precipitation in purifying a virus. In the following pages we are reporting electrophoretic studies made on the virus causing tobacco mosaic.

## EXPERIMENTAL WORK

*Methods.*—For the purpose of our experiment the Todd[64] U tube electrophoresis apparatus was selected. To prevent polarization of the electrodes, agar bridges filled with 1 per cent KCl in 2 per cent agar connected each limb of the U tube to electrode vessels containing a saturated solution of $CuSO_4$. Strips of copper plate were used for electrodes.

Buffer solutions of pH 9 to pH 3 were made according to Clark.[17] Those having pH below 3 were made of potassium pthalate, of phosphoric acid adjusted with hydrochloric acid, or contained only hydrochloric acid.

The plant extract containing the virus was derived from tobacco plants showing characteristic mosaic symptoms. The original virus was kindly supplied by Dr. James Johnson of the University of Wisconsin. A meat grinder was used to crush the leaves and the juice was expressed by pressing the pulp in a piece of cotton cloth. The extract was then centrifuged, passed through a Berkefeld "V" candle under aseptic conditions, and the filtrate collected in sterile test tubes. The pH of this filtrate was found to be 5.6.

Extreme care was taken to adjust the filtrate to the same pH as the buffer in the two arms of the apparatus. This was done by adding approximately one part of the buffer solution of the desired pH to two parts of the plant extract and then dropping small quantities of 1/5 normal HCl or NaOH into the solution from a fine pipette until the pH was adjusted.

In the first set of experiments, the results of which are shown in table 1, a direct current circuit giving 108 volts was used. The current varied from 4 to 9.5 milliamperes and the time from 3 to 5 hours. In later experiments, the results of which are shown in table 2, two or three radio "B" batteries giving 96 to 145 volts supplied the current, which varied from 6 to 9 milliamperes. The duration of electrophoresis in these experiments was 5 to 7.5 hours.

After electrophoresis, samples were taken from each limb of the U tube. These samples are designated in the tables as "anode" and "cathode" respectively. "Bulb" refers to the sample of adjusted plant extract taken from the central bulb and serves as a check on the activity of the virus. A portion of each of these samples was then tested to determine the change in pH during the course of the experiment. All determinations were made by means of the hydrogen electrode except at pH 1.2. At this pH the hydrogen electrode was found to give erratic results and the colorimetric method was therefore substituted. The remainder of each sample was used for inoculating tobacco plants.

Inoculations in the first set of experiments were made by the ordinary needle puncture method. In the second set young leaves of the tobacco plant were rubbed with a small piece of cotton cloth saturated with inoculum. The latter method was found to give much more consistent results.

*Results.*—The results of the experiments are shown in tables 1 and 2. These results indicate that the virus carries a negative charge when in solutions having a pH between 4 and 9 but that the virus is either uncharged or has a very low P.D. when in solutions having a pH between 3 and 1.2.

Only one plant inoculated with liquid from the cathode developed mosaic symptoms. Since the virus migrated to the anode so consistently at pH 4 and failed to migrate to the cathode at all lower pH values it appears that the infection of this plant must have been accidental. In fact, it is rather surprising that more inconsistencies did not arise since the mosaic disease of tobacco is so easily transmitted during watering and handling of the plants.

TABLE 1

INFLUENCE OF THE pH ON THE DIRECTION OF MIGRATION OF TOBACCO MOSAIC VIRUS; FIRST EXPERIMENT

| *pH* before electrophoresis | Inoculum | Milliamperes | Volts | Time | *pH* after electrophoresis | Number of plants inoculated | Number of plants infected |
|---|---|---|---|---|---|---|---|
| | | | | *hours* | | | |
| 1.0 | Cathode | 6–8 | 108 | 4 | 1.15 | 4 | 0 |
| 1.0 | Anode | 6–8 | 108 | 4 | 1.3 | 4 | 0 |
| 1.0 | Bulb | 6–8 | 108 | 4 | ..... | 2 | 1 |
| 2.0 | Cathode | 5.0 | 108 | 4 | 2.0 | 4 | 0 |
| 2.0 | Anode | 5.0 | 108 | 4 | 2.2 | 4 | 0 |
| 2.0 | Bulb | 5.0 | 108 | 4 | ..... | 2 | 0 |
| 3.0 | Cathode | 4.0 | 108 | 4 | 3.0 | 4 | 0 |
| 3.0 | Anode | 4.0 | 108 | 4 | 3.0 | 4 | 0 |
| 3.0 | Bulb | 4.0 | 108 | 4 | ..... | 2 | 0 |
| 4.0 | Cathode | 6.5 | 108 | 5 | 4.05 | 4 | 0 |
| 4.0 | Anode | 6.5 | 108 | 5 | 4.1 | 4 | 0 |
| 4.0 | Bulb | 6.5 | 108 | 5 | ..... | 2 | 0 |
| 4.0 | Cathode | 4.0 | 108 | 4 | ..... | 4 | 1 |
| 4.0 | Anode | 4.0 | 108 | 4 | ..... | 4 | 0 |
| 4.0 | Bulb | 4.0 | 108 | 4 | ..... | 2 | 1 |
| 5.0 | Cathode | 7.5 | 108 | 5 | 5.0 | 4 | 0 |
| 5.0 | Anode | 7.5 | 108 | 5 | 5.0 | 4 | 2 |
| 5.0 | Bulb | 7.5 | 108 | 5 | ..... | 2 | 1 |
| 6.0 | Cathode | 6.5 | 108 | 3 | 6.2 | 4 | 0 |
| 6.0 | Anode | 6.5 | 108 | 3 | 6.2 | 4 | 3 |
| 6.0 | Bulb | 6.5 | 108 | 3 | 6.2 | 2 | 2 |
| 7.0 | Cathode | 7.5 | 108 | 5 | 7.0 | 4 | 0 |
| 7.0 | Anode | 7.5 | 108 | 5 | 7.2 | 4 | 2 |
| 7.0 | Bulb | 7.5 | 108 | 5 | ..... | 2 | 1 |
| 8.0 | Cathode | 6.75 | 108 | 3 | 8.2 | 4 | 0 |
| 8.0 | Anode | 6.75 | 108 | 3 | 8.2 | 4 | 1 |
| 8.0 | Bulb | 6.75 | 108 | 3 | 7.8 | 2 | 1 |
| 9.0 | Cathode | 9.5 | 108 | 5 | 9.0 | 4 | 0 |
| 9.0 | Anode | 9.5 | 108 | 5 | 9.0 | 4 | 2 |
| 9.0 | Bulb | 9.5 | 108 | 5 | 9.0 | 2 | 1 |

TABLE 2

INFLUENCE OF THE pH ON THE DIRECTION OF MIGRATION OF TOBACCO MOSAIC VIRUS; SECOND EXPERIMENT

| *pH* before electro-phoresis | Inoculum | Milli-amperes | Volts | Time | *pH* after electro-phoresis | Number of plants inoculated | Number of plants infected |
|---|---|---|---|---|---|---|---|
| | | | | *hours* | | | |
| 1.2 | Cathode | 9.0 | 96 | 7.5 | 1.3 | 10 | 0 |
| 1.2 | Anode | 9.0 | 96 | 7.5 | 2.0 | 10 | 0 |
| 1.2 | Bulb | 9.0 | 96 | 7.5 | 1.4 | 10 | 10 |
| 1.8 | Cathode | 8.0 | 145 | 5.0 | 2.1 | 5 | 0 |
| 1.8 | Anode | 8.0 | 145 | 5.0 | 2.6 | 5 | 0 |
| 1.8 | Bulb | 8.0 | 145 | 5.0 | 2.1 | 2 | 2 |
| 2.1 | Cathode | 8.5 | 145 | 7.5 | 2.5 | 5 | 0 |
| 2.1 | Anode | 8.5 | 145 | 7.5 | 2.8 | 5 | 0 |
| 2.1 | Bulb | 8.5 | 145 | 7.5 | 2.55 | 2 | 2 |
| 3.0 | Cathode | 6.0 | 96 | 7.5 | 3.0 | 5 | 0 |
| 3.0 | Anode | 6.0 | 96 | 7.5 | 3.05 | 5 | 0 |
| 3.0 | Bulb | 6.0 | 96 | 7.5 | 3.2 | 2 | 2 |
| 4.0 | Cathode | 7.0 | 145 | 7.5 | 4.05 | 5 | 0 |
| 4.0 | Anode | 7.0 | 145 | 7.5 | 4.1 | 5 | 5 |
| 4.0 | Bulb | 7.0 | 145 | 7.5 | 4.1 | 2 | 2 |
| 4.5 | Cathode | 7.5 | 145 | 7.5 | 4.49 | 5 | 0 |
| 4.5 | Anode | 7.5 | 145 | 7.5 | 4.5 | 5 | 5 |
| 4.5 | Bulb | 7.5 | 145 | 7.5 | 4.5 | 2 | 2 |
| 5.0 | Cathode | 7.5 | 145 | 7.5 | 4.9 | 5 | 0 |
| 5.0 | Anode | 7.5 | 145 | 7.5 | 5.1 | 5 | 5 |
| 5.0 | Bulb | 7.5 | 145 | 7.5 | 5.0 | 2 | 2 |
| 6.0 | Cathode | 8.0 | 140 | 5.5 | 5.95 | 5 | 0 |
| 6.0 | Anode | 8.0 | 140 | 5.5 | 5.90 | 5 | 5 |
| 6.0 | Bulb | 8.0 | 140 | 5.5 | 6.0 | 2 | 2 |
| 8.0 | Cathode | 8.0 | 145 | 5.0 | 6.8 | 5 | 0 |
| 8.0 | Anode | 8.0 | 145 | 5.0 | 7.0 | 5 | 5 |
| 8.0 | Bulb | 8.0 | 145 | 5.0 | 7.0 | 2 | 2 |
| 9.0 | Cathode | 8.0 | 145 | 5.0 | 8.90 | 5 | 0 |
| 9.0 | Anode | 8.0 | 145 | 5.0 | 8.95 | 5 | 5 |
| 9.0 | Bulb | 8.0 | 145 | 5.0 | 9.0 | 2 | 2 |

## DISCUSSION

From the results obtained it appears that electrophoresis at pH 4 should separate the virus from at least some of the proteins present in the plant extract. Cohn, Gross, and Johnson[(19)] gave evidence that certain of the proteins in tomato juice have an isoelectric point around pH 5 and it is probable that some of the proteins in tobacco also have isoelectric points above pH 4. Such proteins would migrate to the cathode at pH 4 and would therefore be separated from the virus, which migrates to the anode at this pH.

The fact that the virus did not migrate to the cathode at low pH values indicates that the virus did not adsorb sufficient protein to modify its electrophoretic behavior and suggests that the observed electrophoretic behavior of the virus is similar to that which would be exhibited by the pure virus.

An electrophoretic behavior such as that observed would be expected if the surface of the virus particles were composed largely of compounds which are weak acids. Such compounds would exist as dissociated salts at high pH values, the anion on the surface of the particle giving it a negative charge. At lower pH values such compounds would be changed to undissociated weak acids which would not confer a charge on the surface of the particles.

According to Bayliss[(7)] "practically all inert substances are negative to pure water. In acid solution they are either positive or less negative than in pure water, while in alkaline solutions the negative charge is increased."

Loeb[(41)] studied the electrophoretic behavior of a number of kinds of chemically inert particles such as mastic, graphite, gold and collodion. In concentrations of HCl below M/128 these particles had a negative charge. In M/128 to M/32 HCl a slight positive charge was produced on mastic, graphite, and gold, but collodion remained negative even in M/8 HCl.

The electrophoretic behavior of bacteria as discussed earlier in the paper is very similar to that of the particles of mastic, graphite, and gold studied by Loeb.

Since viruses and bacteriophages have been considered by numerous workers to be non-living and enzymic in nature it may be of interest to briefly discuss the evidence upon this point which is afforded by the electrophoretic behavior of enzymes. According to

Waksman and Davison[66] invertase is isoelectric at pH 5, catalase at pH 7, trypsin at pH 10.2. These isoelectric points are all higher than is usually found for bacteria.

Tobacco mosaic virus exhibited an electrophoretic behavior very similar to that of bacteria and chemically inert particles, the only difference being in the failure of the virus to migrate to the cathode at pH values below 3.

The failure of the virus to migrate to the cathode at low pH values is of questionable significance. Most bacteria that have been studied have carried a relatively weak positive charge at pH values around 1 to 3. Winslow, Falk, and Caulfield[69] found that the most rapid movement of *B. cereus* toward the cathode was 2.8 microns per second at pH 1.5 while the most rapid migration toward the anode was at pH 7.2 when a migration velocity of 10.6 microns per second was attained. It is possible that the virus may have carried a weak positive charge at certain of the pH values at which it appeared to be isoelectric but that its rate of migration was so slow that it was not detected.

Several workers have shown that the migration of bacteria toward the cathode may be inhibited by certain salts in the solution. For example, Winslow, Falk, and Caulfield[69] found that the addition of .363M NaCl prevented the migration of *B. cereus* toward the cathode. Thus it appears that the potential at the surface of bacteria when they tend to migrate toward the cathode is so slight as to be easily inhibited by various constituents in the surrounding medium. It therefore appears that we cannot consider the failure of tobacco mosaic to migrate to the cathode as evidence that it is of a different nature from bacteria.

Our results do not agree with those of Vinson and Petre,[65] who reported that tobacco mosaic virus migrated to the negative electrode when contained in a concentrated tomato extract having a pH of 4.76. The reason for this difference in results is not evident.

## SUMMARY

Unpurified tobacco mosaic virus migrated to the anode during electrophoresis between pH 4 and pH 9. No migration of the virus was detected between pH 3 and 1.2.

## LITERATURE CITED

[1] Allard, H. A.
1914. The mosaic diseases of tobacco. U. S. Dept. Agr. Bul. **40**:1–34.

[2] Allard, H. A.
1915. Effect of dilution on the infectivity of the mosaic disease of tobacco. Jour. Agr. Res. **3**:295–299.

[3] Allard, H. A.
1916. Some properties of the virus of the mosaic disease of tobacco. Jour. Agr. Res. **6**:649–672.

[4] Arnold, L., and E. Weiss.
1925 Isolation of bacteriophage free from bacterial proteins. Jour. Infect. Dis. **37**:411–417.

[5] Arthur, J. M., and J. M. Newell.
1929. The killing of plant tissue and the inactivation of tobacco mosaic virus by ultra-violet radiation. Amer. Jour. Bot. **16**:338–353.

[6] Baker, S. L., and S. H. Nanavutty.
1929. A quantitative study of the effect of ultra-violet rays upon bacteriophage. Brit. Jour. Exp. Path. **10**:45–60.

[7] Bayliss, W. M.
1924. Principles of general physiology. Fourth ed. Longmans, Green, and Co., London.

[8] Beijerinck, M. W.
1899. Ueber ein Contagium vivum fluidum als Ursache der Fleckenkrankheit der Tabaksblaetter. Centbl. Bakt. **5**:27–33.

[9] Bertrand, G.
1904. Etude biochimique de la bacterie du sorbose. Ann. Chim. et Phys. **3**:181–288.

[10] Bertrand, G., and P. Thomas.
1920. Practical biological chemistry. 348 pp. G. Bell and Sons, Ltd., London.

[11] Bovie, W. T.
1915. The action of light on protoplasm. Amer. Jour. Trop. Dis. and Prev. Med. **2**:506–517.

[12] Brewer, P. H., H. R. Kraybill, and M. W. Gardner.
1927. Purification of the virus of tomato mosaic. Phytopath. **17**:744.

[13] Bronfenbrenner, J.
1926. Does bacteriophage respire? Science **63**:51–52.

[14] Bronfenbrenner, J.
1926–27. Further evidence of the resistance of bacteriophage to alcohol. Proc. Soc. Exper. Biol. **24**:373.

[15] Carsner, E.
1925. Attenuation of virus of sugar beet curly-top. Phytopath. 15:745–758.

[16] Chapman, G. H.
1917. Mosaic disease of tobacco. Massachusetts Agr. Exp. Sta. Bul. 175:73–117.

[17] Clark, W. M.
1920. The determination of hydrogen ions. 317 pp. Williams and Wilkins Company, Baltimore.

[18] Cleveland, L. R.
1926. Some problems which may be studied by oxygenation. Science 63:168–170.

[19] Cohn, E. J., J. Gross, and O. C. Johnson.
1919–20. Isoelectric point of the proteins in certain vegetable juices. Jour. Gen. Physiol. 2:145–160.

[20] De Groat, A. F.
1927. The bacteriophage: A method of isolation. Jour. Immunol. 14:175–179.

[21] Dickson, B. T.
1922. Studies concerning mosaic diseases. McDonald College Tech. Bul. 2:1–125.

[22] Douglas, S. R., and Wilson Smith.
1928. Cataphoresis experiment with the virus of vaccinia. Brit. Jour. Exp. Path. 9:213–215.

[23] Duggar, B. M., and J. L. Karrer.
1921. The size of the infective particles in the mosaic disease of tobacco. Ann. Missouri Bot. Gard. 8:343–356.

[24] Duggar, B. M., and J. K. Armstrong.
1925. The effect of treating the virus of tobacco mosaic with the juices of various plants. Ann. Missouri Bot. Gard. 12:359–366.

[25] Elmer, O. H.
1926. Inhibition of mosaic infection. Phytopath. 16:67–68.

[26] Esty, R. J.
1922. Heat resistance of *B. botulinus* spores. Abstracts Bact. 7:6.

[27] Falk, I. S., and M. A. Jacobson.
1926. Electrophoretic potental, acid and serum agglutination of pneumococci. Jour. Infect. Dis. 38:182–187.

[28] Falk, I. S., C. G. Sharp, and G. K. K. Link.
1926–27. Relation between pH, agglutination and P. D. with *Bacterium phaseoli sojense*. Proc. Soc. Exp. Biol. and Med. 24:576–578.

[29] Goldsworthy, M. C.
1926. Attempts to cultivate the tobacco mosaic virus. Phytopath. 17:873–875.

[30] Goldstein, Bessie.
1924. Cytological studies of living cells of tobacco plants affected with mosaic disease. Torrey Bot. Club Bul. 51:261–273.

[31] Hastings, E. G.
1922. A comparison between the resistance to an unfavorable environment of organisms that have grown in native habitat and the same kind grown in artificial culture. Abstracts Bact. **7**:6.

[32] Hoggan, I. A.
1927. Cytological studies on virus diseases of solanaceous plants. Jour. Agr. Res. **35**:651–671.

[33] Holmes, F. O.
1928. Cytological study of the intracellular body characteristic of Hippeastrum mosaic. Bot. Gaz. **86**:50–57.

[34] Iwanowski, D.
1893. Ueber zwei Krankheiten der Tabakspflanze. (Abstr.) Beih. Bot. Centbl. **3**:266–268.

[35] Iwanowski, D.
1903. Ueber die Mosaikkrankheit der Tabakspflanze. Zeitschr. Pflanzenkrank. **13**:1–44.

[36] Johnson, J.
1926. The attenuation of plant viruses and the inactivating influence of oxygen. Science **64**:210.

[37] Johnson, J.
1928. Further studies on the attenuation of plant viruses. Phytopath. **18**:156.

[38] Kunkel, L. O.
1924. Histological and cytological studies on the Fiji disease of sugar cane. Hawaiian Sugar Planters Assoc. Bul. Exp. Sta. Bot. Ser. **3**:99–107.

[39] Kunkel, L. O.
1926. Studies on aster yellows. Amer. Jour. Bot. **13**:646–705.

[40] Loeb, J.
1923–24. On the location of the forces which determine the electrical double layer between collodion particles and water. Jour. Gen. Physiol. **6**:105–129.

[41] Loeb, J.
1923–24. The influence of the chemical nature of solid particles on their cataphoretic P. D. in aqueous solutions. Jour. Gen. Physiol. **6**:215–237.

[42] Mayer, A.
1886. Ueber die Mosaikkrankheit des Tabaks. Die Landwirtschaftlichen Versuchs-Stationen **38**:450–467. Abstract in Jour. Mycol. **7**:382–385.

[43] McKinley, E. B., R. Fisher, and M. Holden.
1925–26. Action of ultra-violet light upon bacteriophage and filterable viruses. Proc. Soc. Exp. Biol. **23**:408–412.

[44] McKinney, H. H.
1927. Factors affecting certain properties of a mosaic virus. Jour. Agr. Res. **35**:1–12.

[45] McKinney, H. H., S. H. Eckerson, and R. W. Webb.
1923. Intracellular bodies associated with a mosaic of *Hippeastrum johnsonii*. Phytopath. **13**:41–47.

[46] MULVANIA, M.
1925. Cultivation of the virus of tobacco mosaic by the method of Olitsky. Science 62:37.

[47] MULVANIA, M.
1926. Studies on the nature of the virus of tobacco mosaic. Phytopath. 16:853–871.

[48] NORTHRUP, J. H., and P. DE KRUIF.
1922. Agglutination of the bacillus of rabbit septicema and of *Bacillus typhosus* by electrolytes. Jour. Gen. Physiol. 4:639–667.

[49] NORTHRUP, J. H., and P. DE KRUIF.
1922. The stability of bacterial suspensions. III. Agglutinations in the presence of proteins, normal serum and immune serum. Jour. Gen. Physiol. 4:655–667.

[50] OLITSKY, P. K.
1924. Experiments on the cultivation of the active agent of tobacco and tomato mosaic. Science 60:593–594.

[51] OLITSKY, P. K., and P. H. LONG.
1929. Isolation by cataphoresis of virus from vaccinia recovered rabbit. Science 49:170.

[52] OLITSKY, P. K., J. TRAUM, and H. W. SCHOENING.
1928. Report of the Foot and Mouth Commission of the U. S. Dept. Agr. Tech. Bul. 76:1–172.

[53] PALLADIN, V. I.
1926. Plant physiology. English translation by B. E. Livingston. P. Blackiston's Son and Co., Philadelphia.

[54] PALM, B. T.
1922. De Mosaiekziekte van de Tabak een Chlamydozoonose. Deli-Proefstation te Medan. Sumatra Bul. 15:1–10.

[55] PRAUSNITZ, C.
1922. Ueber die Natur des d'Herelleschen Phaenomens. Klin. Wochnschr. 1:1639.

[56] PURDY, HELEN A.
1926. Attempts to cultivate an organism from tomato mosaic. Bot. Gaz. 81:210–217.

[57] PURDY, HELEN A.
1928. Multiplication of the virus of tobacco mosaic in detached leaves. Amer. Jour. Bot. 15:94–99.

[58] RAWLINS, T. E., and JAMES JOHNSON.
1924. Cytological studies of the mosaic disease of tobacco. Amer. Jour. Bot. 12:19–23.

[59] SHERMAN, H. C., M. L. CALDWELL, and M. ADAMS.
1926. Further experiments on the purification of pancreatic amylase. Proc. Soc. Exp. Biol. and Med. 23:413–416.

[60] SEVERIN, H. H. P.
1921. Minimum incubation period of causative agent of curly-leaf in beet leaf hopper and sugar beets. Phytopath. 11:424–429.

[61] Smith, F. F.
1927. Some cytological and physiological studies of mosaic diseases and leaf variations. Ann. Missouri Bot. Gard. **13**:425–484.

[62] Smith, J. H.
1928. Experiments with a mosaic disease of tomato. Ann. Applied Biol. **15**:155–167.

[63] Smith, R. E., and P. A. Boncquet.
1915. Connection of a bacterial organism with curly-leaf of sugar beet. Phytopath. **5**:335–342.

[64] Todd, C.
1927. On the electrical behavior of the bacteriophage. Brit. Jour. Exp. Path. **8**:369–376.

[65] Vinson, C. G., and A. W. Petre.
1929. Mosaic disease of tobacco: purification. Bot. Gaz. **87**:14–37.

[66] Waksman, S. A., and Davison, W. C.
1926. Enzymes. Williams and Wilkins Co., Baltimore.

[67] Walker, M. N.
1926. A comparative study of the mosaic diseases of cucumber, tomato and Physalis. Phytopath. **16**:431–458.

[68] Willstatter, R., J. Graser, and R. Kuhn.
1922. Zur Kenntnis des Invertins. Hoppe Seylers Zeitschrift fuer Physiologische Chemie. **123**:1–78.

[69] Winslow, C. E. A., I. S. Falk, and M. F. Caulfield.
1924. Electrophoresis of bacteria as influence by hydrogen ion concentration and the presence of sodium and calcium salts. Jour. Gen. Physiol. **6**:177–200.

[70] Winslow, C. E. A., and H. J. Shaughnessy.
1924. The alkaline isopotential point of bacterial cells. Jour. Gen. Physiol. **6**:697–701.